The Titration Curve of Gelatine.—Report to the Medical Research Council.

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(Communicated by Prof. F. G. Hopkins, F.R.S. Received September 26, 1921.)

I. INTRODUCTION.

During the course of some work on the swelling of gelatine gels in acid and alkaline solutions, it became increasingly evident that no fully satisfactory theory of swelling could be postulated until further information had been obtained as to the quantitative relations, both general and particular, holding between the gelatine base and its combined acid in acid systems, and between the gelatine acid and its combined base in alkaline ones respectively. The work described in the following paper is an attempt to study the problem in its simplest form; namely, in a fluid system in which all the components are in solution. Hydrochloric acid and sodium hydroxide were chosen as the acid and base to use in the investigation, since both form highly ionised solutions in water, and since none of the ions resulting carry more than a single charge, thus simplifying any considerations deduced from the law of mass action. It is still an open question how far the law of mass action can be applied to colloidal solutions. It has been shown by Procter (20), Procter and Wilson (21), Wintgen and Kruger (27), that the quantitative relations found by them to exist in the combination of hydrochloric acid with gelatine, under the conditions of their experiments, fell within the general statement of the law, such combination being regarded as a simple case of salt formation.

Procter considered that his results were explicable on two hypotheses:—

(1) That gelatine had a molecular weight of 839, and combined with one molecule of hydrochloric acid to form gelatine hydrochloride; or

(2) That if gelatine had a larger molecular weight, some multiple of 839, say $839x$, then the gelatine molecule combined with x molecules of hydrochloric acid and the ionisation constants of the x hydroxyl groups involved must be the same. The second hypothesis he rejected as improbable.

We still consider that the molecular weight of gelatine must be greater than Procter has allowed, and we do not regard it as improbable that a number of the hydroxyl ions of the gelatine may have approximately equal ionisation constants. Our experimental results suggest that, up to a given

concentration of hydrogen ions, a group of hydroxyl ions having approximately equal ionisation constants is involved; beyond this concentration, and up to a second fixed value, a second group approximating to a second constant is involved; and beyond this again there is slight evidence of a third group. The factors required in order to bring the second and possible third groups into conformity with the generalised statement of the law of mass action are not yet fully known.

II. Material and Method.

The gelatine used in this investigation was Coignet's Gold Label gelatine, purchased in 1914. It was purified by prolonged dialysis in dilute hydrochloric acid at a reaction of $C_H = 10^{-4 \cdot 6}$, and subsequent precipitation in strong alcohol. Details of the purification are given elsewhere (Jordan Lloyd, 7). It was dried with absolute alcohol and ether, and kept in a desiccator over pure sulphuric acid. (It is possible to cause the gelatine to lose more water by heating to 100° over phosphorus pentoxide *in vacuo*.) When dried with absolute alcohol it is a white brittle substance, fibrous in appearance, and containing 0·00 to 0·06 per cent. of ash. It forms clear solutions in water, which set to opaque white gels on cooling. The clear solutions set to colourless, glassy, transparent gels in the presence of free acid or base. The solution referred to below as 1 per cent. gelatine contains 1 grm. of this purified dry gelatine in 100 c.c. of freshly-boiled distilled water at room temperature.

The object of our experiments was to determine the amount of hydrochloric acid or sodium hydroxide which would combine with a constant weight of gelatine, and the method employed throughout was the electrical measurement of the concentration of the free hydrogen ions in solutions containing 1 per cent. of gelatine and known concentrations of hydrochloric acid or sodium hydroxide. The change of hydrogen ion concentration from that of an equally concentrated system containing no gelatine, is a measure of the acid (or base) which has combined with the gelatine. The routine method employed was to take 5 c.c. of a freshly made solution of 2 per cent. gelatine which had cooled but not yet set; the requisite amount of acid (or base) was added from a pipette calibrated to 0·01 c.c., and sufficient freshly-boiled distilled water to make the final volume 10 c.c. The reaction was taken at once by means of a gas chain. A Tinsley potentiometer was used.

The electrodes used were of a very simple modified Barendrecht type, and were made for us by Mr. A. W. Hall, of the Biochemical Laboratory. Their great advantage is the ease with which they are cleaned, a matter of great importance, as we found it essential to clean and re-coat the electrodes after

every reading. A diagram of the electrodes is given below (fig. 1), and is self-explanatory.

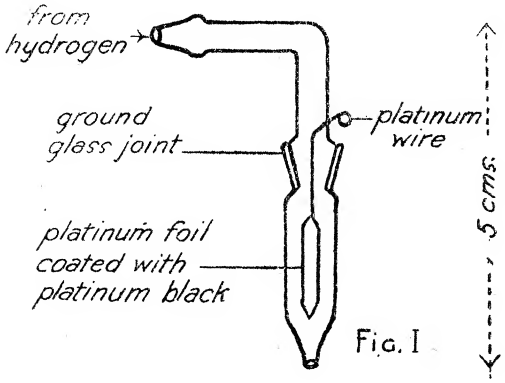


Fig. I

The platinum foil was coated very thinly with platinum black according to the directions of Michaelis (16). Only sufficient platinum was deposited to hide the glint of the foil. With such electrodes it was possible to take readings immediately contact had been made with the experimental fluid This is an essential condition for accuracy. With a slow electrode and a delayed reading, values tend to be very irregular. This is particularly marked in alkaline solutions. The experimental fluid was placed in silica cups into which the electrodes dipped. Contact was made by means of a sliding joint. The solutions had a strong tendency to froth near the iso-electric point. All experimental readings were taken at 20° C.

III. Experimental Results.

(a) *The Gelatine-Hydrochloric Acid Curve.*

If N represents the normality of a solution of hydrochloric acid and α its degree of ionisation, the concentration of the hydrogen ion present may be represented as $N\alpha$ on the normality scale, or $-\log N\alpha$ on the logarithmic scale. If 1 per cent. of gelatine be introduced into such a system, the reaction (P_H) is no longer given by the expression $-\log N\alpha$, but by some lower value. This can be determined by the hydrogen electrode.

The following experimental values were obtained for the variation of reaction (P_H) with total acid-content (N), gelatine concentration being kept constant at 1 per cent. temperature at 20° C. The constants used in calculating the reaction (P_H) from the observed electromotive force (E) are taken from Michaelis' 'Wasserstoffionen Konzentration' (16). The values for the concentration of the hydrogen ion are given on both the logarithmic scale P_H, and in terms of normality [H]. From the values of [H] it is possible to calculate the concentration of un-ionised free hydrochloric acid present in the

system. If this is represented as [HCl], and α is the degree of ionisation of the acid, then

$$[\mathrm{HCl}] = [\mathrm{H}]/\alpha - [\mathrm{H}]. \qquad (1)$$

The values of α are taken from Lewis's 'Text-book of Physical Chemistry' (3rd edition). Using Blasel and Matula's formula (1), if n' represents the concentration of acid removed from independent solution by the gelatine, then

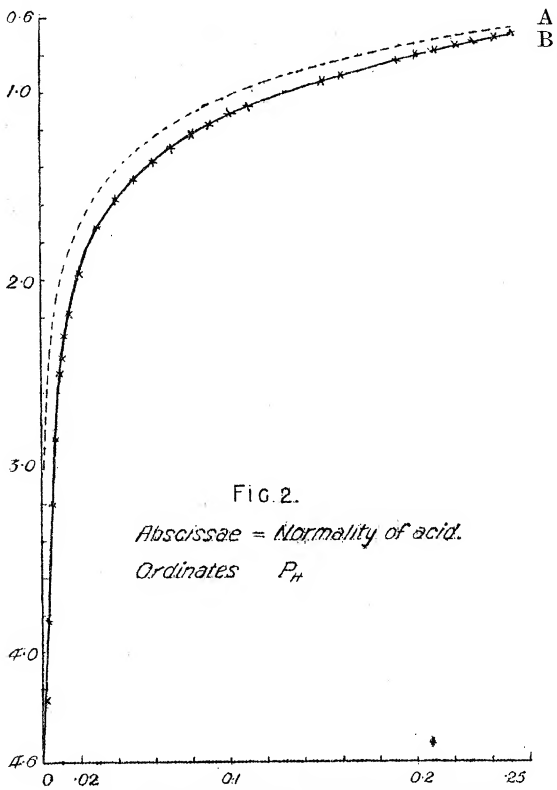
$$n' = \mathrm{N} - [\mathrm{H}]/\alpha, \qquad \text{or} \qquad n' = \mathrm{N} - ([\mathrm{H}] + [\mathrm{HCl}]). \qquad (2)$$

Equation (2) only holds if the value of α in (1) is the same both in the presence and absence of dissolved gelatine. In Table I, Column I gives the concentration of acid used; Column II the corresponding values of α, and Column III the values of $-\log \mathrm{N}\alpha$. Column IV gives the electrode reading in millivolts, and V the value of $\mathrm{P_H}$ calculated from the formula

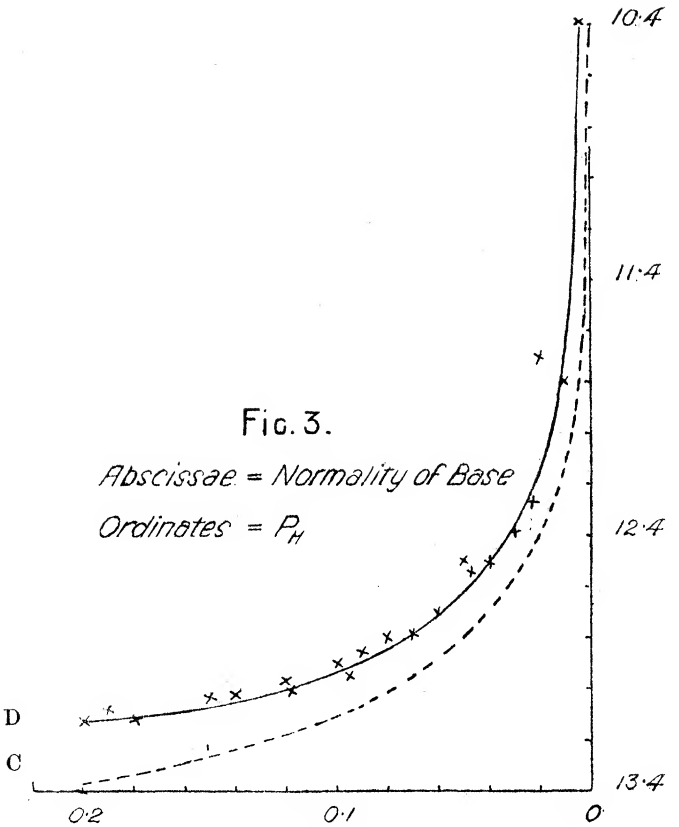
$$\mathrm{P_H} = (\mathrm{E} - 248{\cdot}8)/58{\cdot}1.$$

Columns VI and VII give [H] and n' respectively.

The two curves $-\log \mathrm{N}\alpha : \mathrm{N}$ and $\mathrm{P_H} : \mathrm{N}$ from the values given in Table I are shown in fig. 2, and are designated as A and B respectively.



Buffer curve of gelatine in hydrochloric acid.



Buffer curve of gelatine in sodium hydroxide.

Blasel and Matula, in calculating n', the hydrochloric acid fixed by gelatine, as $n' = N - [H]/\alpha$, assume that the value for α is the same when $[H] = [Cl]$ as when $[H] \neq [Cl]$, provided that the values for [H] are equal. This, however, is not true. A closer approximation is obtained by taking the square root of the product of [H] and [Cl] as the basis of the calculation. The value for [H] is obtained experimentally from hydrogen-electrode readings. The value for [Cl] is obtained by assuming that the gelatine chloride is completely ionised, and that therefore, $[Cl] = [H] + n'$. This assumption is also made by Procter and Wilson (21), though Bugarsky and Liebermann's experimental figures on the concentration of the chlorine ions do not support it (3). The amount of un-ionised acid [HCl] depends, therefore, not upon [H] but on $[H]_{corr.}$, where

$$[H]_{corr.} = \sqrt{([H] \times [Cl])},$$

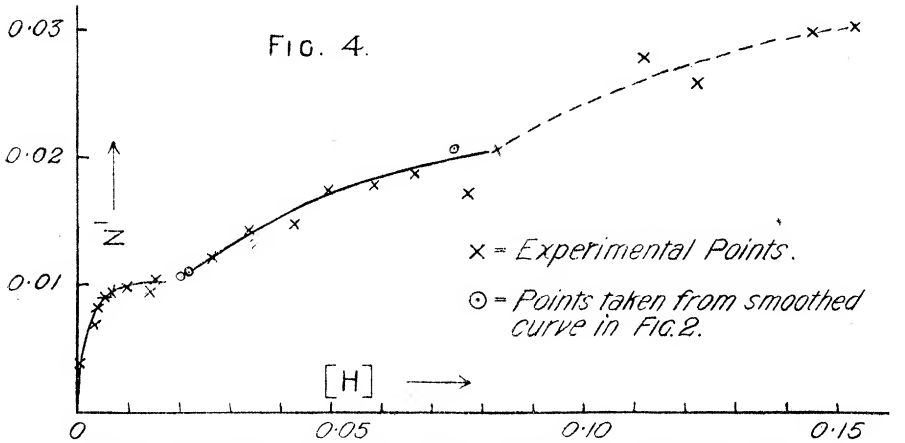
and a new value for the acid fixed, say N′, follows as before. This is given in Column VIII and is shown plotted in fig. 4. [In the values for n' calculated

Table I.

I. Concentration of HCl = N.	II. Degree of ionisation = α.	III. $-\log N\alpha$.	IV. E.M.F. in millivolts of gas chain of 1 per cent. gel. solution = E.	V. $P_H = E - 248{\cdot}8/58{\cdot}1$.	VI. [H.]	VII. $n' = N - [H]/\alpha$.	VIII. $N' = N - [H]_{corr.}/\alpha$.
0·0000		7·06	548·2	5·151			
0·0040	0·980	2·39	471·5	3·833	0·00015	0·00385	0·00383
0·0101	0·972	2·00	394·0	2·500	0·00316	0·00686	0·00679
0·0121	0·969	1·94	389·5	2·420	0·00380	0·00821	0·00812
0·0141	0·967	1·88	383·0	2·310	0·00490	0·00910	0·00898
0·0161	0·965	1·83	376·1	2·191	0·00644	0·00952	0·00939
0·0200	0·962	1·72	365·3	2·005	0·00988	0·00984	0·00970
0·02417	0·958	1·63	356·2	1·848	0·01419	0·00960	0·00946
0·0300	0·953	1·54	349·0	1·720	0·01514	0·0105	0·0104
0·0320				*1·69*	*0·0204*	*0·0108*	*0·0107*
0·0340				*1·66*	*0·0219*	*0·0112*	*0·0110*
0·0403	0·949	1·42	340·2	1·573	0·0267	0·0124	0·0120
0·0503	0·944	1·32	334·2	1·470	0·0339	0·0148	0·0142
0·0503				*1·455*	*0·0350*	*0·0135*	*0·0129*
0·0604	0·939	1·25	328·3	1·369	0·0428	0·0152	0·0147
0·0705	0·935	1·18	324·6	1·305	0·0496	0·0179	0·0173
0·0806	0·931	1·12	320·5	1·234	0·0583	0·0185	0·0177
0·0906	0·928	1·07	317·2	1·177	0·0665	0·0195	0·0186
0·1007	0·923	1·03	313·5	1·114	0·0769	0·0180	0·0171
0·1007				*1·13*	*0·0741*	*0·0215*	*0·0205*
0·1108	0·922	0·95	311·7	1·082	0·0828	0·0216	0·0206
0·1510	0·912	0·86	304·1	0·952	0·1117	0·0295	0·0278
0·1611	0·910	0·83	301·8	0·912	0·1225	0·0274	0·0258
0·1913	0·905	0·77	297·5	0·838	0·1452	0·0319	0·0298
0·2014	0·903	0·74	296·1	0·814	0·1535	0·0324	0·0302
0·2115	0·902	0·72	294·2	0·782	0·165	0·029	0·027
0·2215			292·4	0·750	0·178	0·024	0·022
0·2316			291·1	0·728	0·187	0·023	0·022
0·2417			290·1	0·711	0·194	0·027	0·025
0·2517			288·6	0·685	0·207	0·023	0·021

N.B.—Figures in italics are taken from the smoothed curve and not from experimental points.

from Blasel and Matula's formula it is assumed that the gelatine hydrochloride contributes no chlorine ions to the system; in those for N′ it is assumed that the gelatine hydrochloride contributes all its chlorine as free



Curve of acid fixed. Abscissæ = [H]. Ordinates = Acid fixed or N′.

chlorine ions to the system. The two curves, n' : [H] and N′ : [H], therefore form the limits within which the actual curve must lie. In hydrochloric acid of concentrations less than 0·02 grm. of free hydrogen ion per litre the difference between the two limiting curves is negligible.] It can be seen that the curve is not a simple smooth curve, but that it consists of two, and possibly three, distinct regions. The deductions from this will be considered after the gelatine-sodium hydroxide curves have been described.

(b) *The Gelatine—Sodium Hydroxide System.*

Let N represent the normality of the caustic soda and α its degree of ionisation at 20° C., then $-\log N\alpha$ equals the hydroxyl ion concentration of the system and $14{\cdot}13-(-\log N\alpha)$ is the hydrogen ion concentration. Values for α are given by Kohlrausch (9), Noyes (18) and Jones (6). Unfortunately they differ considerably. The values given by Noyes were taken to plot the (broken) curve $14{\cdot}13+\log N\alpha$ in fig. 3 (marked C in the margin). Noyes' values only go to a concentration 0·05 N. The curve was extended beyond this region by taking Jones's figures for α and adding to them the difference between his figures and Noyes', which may be taken as 0·05 if α is expressed as fractions of unity. N, α and $14{\cdot}13+\log N\alpha$ are shown in the first three columns of Table II. The fourth column gives the readings obtained for E, the electro-motive force at the surface of the hydrogen electrode in a 1·00 per cent. solution of gelatine. The fifth column,

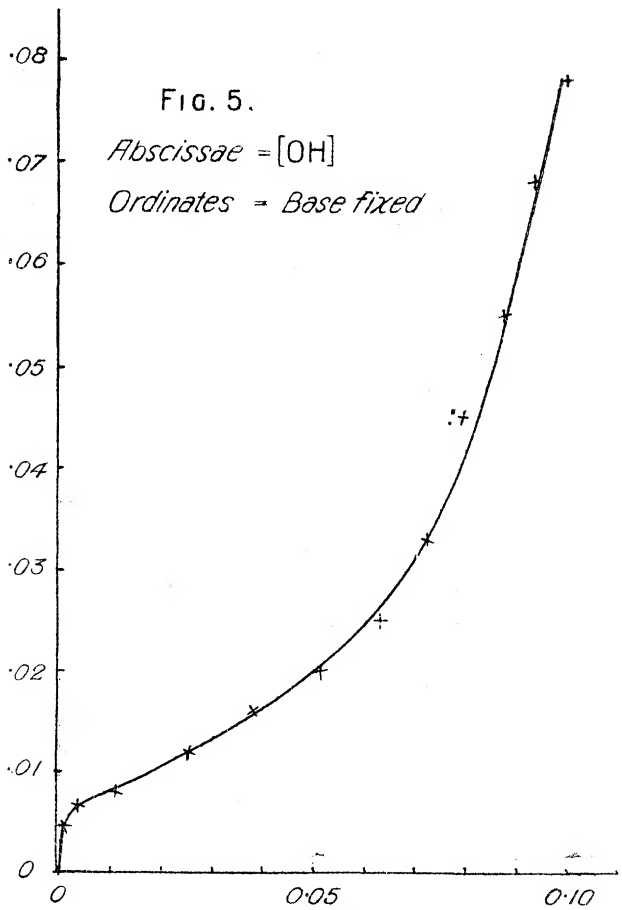
Table II.

NaOH = N.	Degree of ionisation = α.	14·13 + log Nα.	E.M.F. = E.	$P_H = E - 248{\cdot}8/58{\cdot}1$. Observed.	$P_H = E - 248{\cdot}8/58{\cdot}1$. From smoothed curve.	[OH] = antilog $-(14{\cdot}13 - P_H)$.	NaOH unfixed = [OH]/α.	$n' = N - [OH]/\alpha$.
0·000			548·2	5·15				
0·002	0·984	11·42	557·2	5·30				
			685·0	7·50				
0·004	0·976	11·72	726·0	8·22				
0·005	0·974	11·82	852·0	10·39				
			969·0	12·40	*10·40*	*0·000*	0·000	*0·005*
0·006	0·972	11·90	786·0	9·24				
0·010	0·962	12·11	934·5	11·80				
			934·5	11·80	*11·65*	*0·003*	0·003	*0·007*
0·020	0·949	12·41	929·0	11·71	*12·17*	*0·111*	0·112	*0·008*
0·023	0·948		961·5	12·26				
0·030	0·939		969·0	12·39				
			952·0	12·06				
			975·5	12·50				
0·040	0·933	12·70	975·5	12·50				
			975·5	12·50	*12·53*	*0·025*	0·028	*0·012*
0·047	0·929		977·2	12·54				
0·050	0·923		975·5	12·50				
			975·5	12·50				
0·060	0·921	12·87	986·1	12·70	*12·71*	*0·038*	0·044	*0·016*
0·070	0·917		991·2	12·78				
			992·2	12·79				
0·080	0·913	12·99	993·5	12·80	*12·84*	*0·051*	0·060	*0·020*
0·090	0·910		995·8	12·85				
0·094	0·909		1001·1	12·95				
0·100	0·908	13·09	995·8	12·85				
			998·2	12·90	*12·93*	*0·063*	0·075	*0·025*
0·117	0·903		1002·5	12·97				
0·120	0·903				*12·99*	*0·073*	0·087	*0·033*
0·140	0·898		1005·6	13·02	*13·03*	*0·079*	0·095	*0·045*
0·150	0·896	13·26	1006·1	13·03				
0·160	0·893				*13·07*	*0·087*	0·105	*0·055*
0·180	0·890				*13·10*	*0·093*	0·112	*0·068*
0·190	0·888		1008·7	13·08				
0·200	0·887	13·37	1014·0	13·16				
			1011·4	13·12	*13·13*	*0·100*	0·122	*0·078*
0·400	0·84		1030·5	13·44		0·204	0·235	0·165
0·500	0·83		1036·8	13·54		0·257	0·300	0·200

N.B.—Figures in italics are taken from the smoothed curve and not from experimental points.

which is sub-divided, gives values for P_H calculated from E, and read from the smoothed curve $N : P_H$ (marked D in fig. 3). The sixth column gives [OH] the concentration of hydroxyl ions on the normality scale, and the final column gives n', which is equated to $N-[OH]/\alpha$, and is the first calculation for the amount of base "fixed" by the gelatine.

In taking the "acid-fixed" values, the calculations in Table I were made directly from the experimental readings of E. These lie so very closely to a smooth curve that it is safe to assume that the experimental error is slight. In the case of the values of E in alkaline solution, the error is obviously very much greater. Chemical destruction of the gelatine by hydroxyl in the presence of spongy platinum is probably the cause, and hydrolysis has also been shown to have a slight effect on the reading. For this reason, an arbitrary smooth curve ($P_H : N$ or D in fig. 3) was drawn through the



Curve of base fixed, n'.

experimental points, and the calculations of n' have been made from P_H readings taken from this smooth curve and not from the P_H readings given in Table II. Taking into consideration the poor quality of our experimental determinations of E in alkaline solutions, and the lack of agreement as to the values of α for caustic soda, it has not been considered worth while to correct the values of n' obtained by the Blasel and Matula formula. Fig. 5 shows n' plotted against [OH], and will be referred to later in Section IV (*b*).

IV. The Calculation of the Ionisation Constants of Gelatine.

(a) *The Value of* K_b.

The combination of gelatine with hydrochloric acid between 0·00 and 0·25 N concentration of acid may be represented as a curve with three sections.

Assuming for the moment that gelatine in hydrochloric acid solution behaves as a monacidic base from 0·00 to 0·04 N HCl, and combines with the acid to form an ionisable salt (see Introduction), the system may be represented as follows:—

Let [G(H)OH] represent the un-ionised gelatine base, and [GH′] and [OH′] the two ions of the ionised base; and assume that the salt GHCl is completely ionised; now by the law of mass action for a weak base,

$$\frac{[GH'] \times [OH']}{[G(H)OH]} = K_b,$$

$$\therefore \frac{[GH']}{[G(H)OH]} = \frac{[H]}{K_1} \text{ where } K_1 = \frac{K_w}{K_b}, \text{ the hydrolysis constant of the base.}$$

Let C = the equivalent concentration of the gelatine.

Then $[GH'] + [G(H)OH] = C$,

$$\therefore \quad \frac{[GH']}{C} = \frac{H}{H+K_1}, \tag{3}$$

or

$$\frac{[GH']}{[GH'] + [G(H)OH]} = \frac{H}{H+K_1}.$$

Now, in equation (1), [GH′] is equal to N′ and is known, H is known, and therefore there are two unknown quantities, C and K_1. If Procter's (20) value of 839 be taken as the reacting weight of gelatine, C = 0·012. Wilson's (26) later value of 768 makes C equal to 0·013. Wintgen and Kruger (27), using the catalysis of methyl acetate as a measure of the concentration of the hydrogen ion, obtain a molecular weight of 839 for gelatine, while, calculating from the experimental results of Pauli and Hirschfeldt, they obtain the value 881·4. They give $2{\cdot}7 \times 10^{-11}$ as a value for K_b at 25° C. In the calculations given below, C is taken as 0·0120.

Substituting for N′, H, G(H)OH in equation (1), we get the following values for K_1:—

N′.	[H.]	[G(H) OH.]	K_1.
0·00679	0·00316	0·00521	0·00242
0·00812	0·00380	0·00388	0·00184
0·00898	0·00490	0·00302	0·00165
0·00939	0·00644	0·00261	0·00180

K_1 may therefore be approximated to 0·0018,

whence $$K_b = \frac{K_w}{K_1} = \frac{10^{-14\cdot1}}{0\cdot0018} = 0\cdot48 \times 10^{-11}. \qquad (4)$$

A comparison of this value with Procter's value of $5\cdot2 \times 10^{-12}$ shows that they are both of the same order. It is a workable hypothesis to suppose that gelatine in the presence of hydrochloric acid, the concentration of which lies between 0·00 and 0·04 N, behaves according to the law of mass action, like a weak base with a reacting weight of 839 and ionisation constant of $4\cdot8 \times 10^{-12}$, each reacting mass combining with one equivalent of acid. Since we do not consider, on the chemical evidence at present available, that the molecular weight of gelatine can be less than 10,000, then it follows that, in its first stage of combination with hydrochloric acid, the gelatine molecule has available thirteen points of attachment for acid, all with a chemical potential very close to $0\cdot48 \times 10^{-11}$. Procter has considered this possibility, and rejects it as improbable, but, if we consider that the acid is attached to the free $-NH_2$ groups of the lysin and arginin, and possibly some other di-amino-acid, then it does not seem so improbable that the ionisation constants of these basic groupings might be of the same order. Kanitz (8) gives the following values for histidin, arginin and lysin :—

	First ionisation constant.	Second ionisation constant.
Histidin	$5\cdot7 \times 10^{-9}$	$5\cdot0 \times 10^{-13}$
Arginin	$1\cdot0 \times 10^{-7}$	$2\cdot2 \times 10^{-12}$
Lysin	$1\cdot0 \times 10^{-7}$	$1\cdot1 \times 10^{-12}$

These values are for the amino-acids in the free state. With arginin and lysin the first and second ionisation constants are of the same order of magnitude, hence it might be expected that even when bound by one amino-group into the protein molecule, the free amino-groups of both acids would have ionisation constants of the same order of magnitude. Evidence for the binding of the acid at these groups is given below.

The curve given by equation (3), *i.e.*, $\frac{[GH']}{C} = \frac{[H]}{[H]+0{\cdot}0018}$ gives a curve in which GH′ increases with [H] and only attains a maximum at infinite concentration of [H]. If we assume an error of 1 in 1000, [H] may be regarded as at infinite concentration when [H] = 0·01 N. The experimental curve, however, continues to rise very rapidly at still higher concentrations of acid. In his earlier papers Procter considers this difficulty and supposes that a second ionisation constant of a lower value also exists. But equations of the form $y = \frac{x}{x+a} + \frac{x}{x+b}$ where a and b are constants, give when plotted for x and y, a curve which rises rapidly at first and later more slowly, and it proved impossible to fit such a curve to the observations on fig. 4. These seem to indicate a maximum about N′ = 0·01, then a gradual rise to a possible maximum about N′ = 0·02 followed by yet another increase in N′. The observations for the latter part of the curve, however, are too uncertain to justify any definite conclusions.

(*b*) *Calculation of* K_a.

If J is the iso-electric point of an amphoteric electrolyte, K_a and K_b, its ionisation constants,

then
$$J = \sqrt{\left(\frac{K_a}{K_b} \cdot K_w\right)},$$

K_w at 20° C = $0{\cdot}86 \times 10^{-14}$, K_b for gelatine at 20° C = $4{\cdot}8 \times 10^{-12}$

$$J = 10^{-4{\cdot}6} \text{ (17)},$$

whence K_a should equal $3{\cdot}5 \times 10^{-7}$.

Now for a weak acid G (OH) H, we have

$$\frac{[H'] \times [G(OH)']}{[G(OH)H]} = K_a.$$

If [G(OH)′] is put equal to n', and C is put equal to [G(OH)′]+[G(OH)H], *i.e.*, to the initial concentration of the gelatine, then

$$\frac{n'}{C-n'} = \frac{K_a}{[H]} = \frac{K_a}{K_w} \cdot [OH].$$

But if 839 is taken as the reacting weight of gelatine, then C = 0·012, hence n' should be less than 0·012. But n' is already greater than 0·012 in 0·020 N sodium hydroxide and as N increases, n' increases, with an ever-increasing rapidity. Hence C cannot be taken as 0·012, but must be considerably greater. That is, the reacting weight in alkaline solution must be less than in acid solution, and hence different linkages must be involved.

If $$\frac{n'}{C-n'} = \frac{K_a}{K_w} \cdot [OH],$$

or $[OH]/K_2$, where $K_2 = K_w/K_a = 0{\cdot}86 \times 10^{-14}/3{\cdot}5 \times 10^{-7} = 2{\cdot}5 \times 10^{-8}$.

Then $C = (K_2 + [OH])n'/[OH]$, and when we substitute the above value for K_2 we see that n' should be nearly equal to C at extremely small concentrations of hydroxyl ion and should then remain appreciably constant. This is not the case in fig. 5; here n appears to rise abruptly to about 0·005 and there seeks a maximum only to commence rising again to give a very steep curve.

Hence it is obvious that in alkaline solution gelatine does not behave simply as a weak acid dissociating in accordance with the law of mass action. It is possible that this abrupt rise accompanies some structural change of the protein molecule such as Dakin had shown to occur in strong alkaline solution (4). It must always be borne in mind that the hydrolysis of the —C(OH):N— groupings with the formation of free carboxylic and amino-groups occurs very rapidly in alkaline solution as measured by formaldehyde titration. Now the hydrolytic breakdown of the gelatine is not accompanied by a greatly increased basic binding power in the system, for a 1 per cent. solution of gelatine in sodium hydroxide having a reaction of $P_H = 12{\cdot}97$ was found after 3 hours at 100° C. to have changed to a reaction of 12·91. Further standing for 48 hours at room temperature was accompanied by a change of reaction to 12·90. This change in reaction corresponds to an increased combination of gelatine and base to the extent of only 0·011 equivalents of sodium hydroxide to 10 grms. of gelatine.

V. Mechanism of Fixation of the Hydrochloric Acid.

The most obvious points of attachment for acids in the gelatine molecule are the free amino-groups, and if hydrochloric acid forms salts with gelatine by addition of these groups, the salts should be regarded as hydrochlorides. Gelatine contains 18·0 per cent. of nitrogen in its total dry weight. According to Van Slyke and Birchard (25) 3·16 per cent. of this 18 per cent. (equal to half the lysin) can be removed as nitrogen gas by the action of nitrous acid, and can therefore be regarded as existing in the molecule in the form of free amino-groups. If these groups are the only ones in the molecule which can bind hydrochloric acid, then the maximum combining power of 10 grains of gelatine should be 0·0039 equivalents. Kossel and Cameron (11) and Kossel and Kellaway (12) have also brought forward evidence to show that proteins such as clupein and salmin, which contain no lysin, but which contain arginin, have a free amino-group in the guanidin nucleus of the arginin they contain.

This group does not give off nitrogen gas under the action of nitrous acid (Van Slyke, 23). Kossel (10) states that the acid binding power of salmin is exactly that of its guanidin groups, and Kossel and Cameron (11) also consider that the free amino-groups of clupein must be those of its guanidin groups. Bracewell (2) considers that in all proteins the mechanism of acid fixation is by means of free amino-groups, and that in proteins such as gelatine, which contain both lysin and arginin, the acid-binding power should be given by the sum of half the lysin nitrogen plus one-quarter of the arginin nitrogen, each nitrogen atom binding one equivalent of acid. From this he calculates that since lysin contains 6·32 per cent. of the total nitrogen, and arginin 14·70, the maximum binding power of gelatine for acids should be 0·00085 equivalents per gramme, *i.e.*, 0·0085 equivalents for 10 grm. By a titrimetric method he finds that 1 grm. of undissolved gelatine powder can remove 0·00070 equivalents of acid from a supernatant solution.

By the method which we have employed in this work the acid-binding power of gelatine, calculated on its lysin and arginin-content (which we find equal to 0·0086 equivalents for 10 grm. of gelatine), lies not at the true maximum of the $N' : [H]$ curve (fig. 4), but close to the first apparent maximum. It is possible, then, that in solutions of hydrochloric acid less than 0·02 N gelatine binds hydrochloric acid by means of its free amino-groups, and that it is the average ionisation constants of these basic groups that is given by the value $4{\cdot}8 \times 10^{-12}$. But with increasing concentration of acid, more acid is bound than can be accounted for on this hypothesis, and it is therefore necessary to consider what part the imino-nitrogen of the peptide linkage (—COHN—) could play. Robertson (22) states that the acid-binding power of proteins is not much increased by hydrolysis, and we have found that the reaction of a 1 per cent. solution of gelatine, which was found to be $P_H = 1{\cdot}13$, had only changed to $P_H = 1{\cdot}12$ after 7 hours at 100° C. This change is of the same order as the experimental error of the method, nevertheless hydrolysis of the gelatine had occurred during the heating in the strong acid solution, as was shown by the fact that the gelling power had been destroyed. It seems, therefore, that the peptide linkage can function as an acid-binding group.

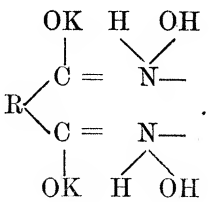
Calculating again from Van Slyke's figures, if every amino-nitrogen atom of the free amino-acids of gelatine (*i.e.*, the amino-nitrogen from lysin and arginin + the imino-nitrogen from the peptide linkages) can act as a point for the fixation of an equivalent of acid, then 10 grm. of gelatine should be able to combine with 0·092 equivalents of hydrochloric acid. If only the di-amino acids (arginin and lysin, together with histidin) can do so, then the maximum value for acid fixed in a 1 per cent. solution of gelatine would be 0·020 equivalents. It can be seen from fig. 3 that the value 0·020 for N— corresponds to

the maximum level of the second branch of the curve. The curve, however, continues to rise to a height of 0·030, though later it falls again. The experimental error in determining P_H becomes enormously magnified on the third limb of the [H] : N curve, and therefore too much stress cannot be laid on its smoothed form. However, it seems clear that some of the —COHN— groups can act as basic groups combining with acids. What *rôle*, if any, other groups (such as the hydroxyl groups of the hydroxy-acids) in the molecule play in acid fixation is still unknown. It will be necessary to follow experimentally the fate of the chlorine ion before final decisions are possible. At present our calculations of N′ involve the assumption that the gelatine hydrochlorides are present as completely ionised salts, an assumption that is liable to lead to an increasing error with increasing values of [H].

The Mechanism for the Fixation of Sodium Hydroxide.

The theory that proteins fix bases by means of their free carboxyl groups has given way on the accumulation of evidence that there are not enough of the latter to explain the quantitative relations

Brailsford Robertson (p. 236) suggests that the enolic imino-linkages $\begin{array}{l}-C=N-\\ \quad\;\;|\\ \quad\;\;OH\end{array}$, are responsible and "that the neutralisation of bases by the proteins is accomplished, at any rate for the greater part, by the dicarboxylic radicles which they contain." He gives the formula for potassium protein compounds as



According to the most recent analysis of gelatine (Dakin, 1920), the only di-carboxy acids present are aspartic acid (3·4 per cent.) and glutamic acid (5·8 per cent.). Calculating from these figures, the maximum combining capacity of a 1 per cent. solution of gelatine should be 0·0168 equivalents. On examining the curve of base fixed in fig. 4, it can be seen that this value for n' is by no means a maximum, but that instead the curve inflects and rises with increasing gradient. If every —COHN— group in the molecule is considered capable of acting as a point of attachment for bases, the maximum value for n' should be 0·09; n' however rises considerably above this figure. There must therefore be other means by which the gelatine molecule can fix sodium hydroxide. The possibility of linkage

at some of the hydroxy-groups of the substituted amino-acids serine and hydroxyproline, is not to be ignored. Hydrolysis of gelatine by caustic soda has been shown to increase slightly its basic binding power, a fact which suggests that not all the —COHN— linkages are as potent as base fixers as the free —COOH— groups. Loeb (14, 15) has shown that bases react with gelatine at the same hydroxyl ion concentrations in equivalent proportions. This fact shows that the reaction is ionic, and that the compounds formed are of the nature of ionisable salts. Loeb only worked with solutions whose alkalinity was less than $P_H = 9$. His experimental values correspond very closely to our values over the same range.

The long slow rise of n' with increasing alkalinity is very striking. A feature in which the alkali-gelatine system differs markedly from the acid-gelatine system is illustrated by a different property of the gelatine, namely, the turbidity of the gel. Sols of 1 per cent. gelatine in distilled water set in a few hours to white, turbid gels. In the presence of 0·001 N hydrochloric acid the gel is not turbid, but quite clear and glassy after 24 hours' standing at 15° C. In the presence of caustic soda, however, the turbidity of the gel 24 hours old persists up to a concentration of 0·002 N soda. Thus there is both quantitative and qualitative evidence to show that in the same protein the mechanism of fixing acids is different from that of fixing bases.

VI. Summary.

1. Hydrochloric acid combines with gelatine in solutions whose acid concentrations are less than 0·04 normal, according to the law of mass action. K_b for gelatine is $4{\cdot}8 \times 10^{-12}$ at 20° C. if 839 be taken as the reacting weight of gelatine.

2. The theory is put forward that over this range of the curve of combination of gelatine with hydrochloric acid, the combination occurs at the free —NH_2 groups. These groups are present in the lysin, arginin, and possibly some other of the amino-acids of the gelatine. The ionisation constants at these groups are taken as approximately equal. The salts formed are hydrochlorides.

3. In concentrations of hydrochloric acid greater than 0·04 normal, the proportion of acid fixed is greater than would follow from the combination of hydrochloric acid with a weak base with an ionisation constant of $4{\cdot}8 \times 10^{-12}$. This is not due to the hydrolytic decomposition of the gelatine and release of further free —NH_2 groups. It seems possible therefore that combination is also occurring at the nitrogen of the peptide linkages.

4. In dilute sodium hydroxide of a concentration less than 0·01 normal, the gelatine combines with the base less rapidly than would follow by a

calculation from the value for K_b and the value for the iso-electric point, if 839 is taken as the reacting weight.

5. It is suggested that the number of positions of attachment for bases is not the same as the number of positions for acids, *i.e.*, that the reacting weight $= \frac{\text{molecular weight}}{\text{basicity (or acidity)}}$ is not the same in acid and alkaline solution.

6. It has not been found possible to calculate a value for K_a.

7. It would appear that in concentrations of sodium hydroxide about 0·1 N the structure of the molecule undergoes some change.

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